Neural induction promotes large-scale chromatin reorganisation of the *Mash1* locus

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Accepted 4 October 2005
Journal of Cell Science 119, 132-140 Published by The Company of Biologists 2006
doi:10.1242/jcs.02727

Summary

Determining how genes are epigenetically regulated to ensure their correct spatial and temporal expression during development is key to our understanding of cell lineage commitment. Here we examined epigenetic changes at an important proneural regulator gene *Mash1* (*Ascl1*), as embryonic stem (ES) cells commit to the neural lineage. In ES cells where the *Mash1* gene is transcriptionally repressed, the locus replicated late in S phase and was preferentially positioned at the nuclear periphery with other late-replicating genes (*Neurod*, *Sprr2a*). This peripheral location was coupled with low levels of histone H3K9 acetylation at the *Mash1* promoter and enhanced H3K27 methylation but surprisingly location was not affected by removal of the Ezh2/Eed HMTase complex or several other chromatin-silencing candidates (G9a, SuV39h-1, Dnmt-1, Dnmt-3a and Dnmt-3b). Upon neural induction however, *Mash1* transcription was upregulated (>100-fold), switched its time of replication from late to early in S phase and relocated towards the interior of the nucleus. This spatial repositioning was selective for neural commitment because *Mash1* was peripheral in ES-derived mesoderm and other non-neural cell types. A bidirectional analysis of replication timing across a 2 Mb region flanking the *Mash1* locus showed that chromatin changes were focused at *Mash1*. These results suggest that *Mash1* is regulated by changes in chromatin structure and location and implicate the nuclear periphery as an important environment for maintaining the undifferentiated state of ES cells.

Key words: Chromatin, Transcription, Replication timing, Nuclear organization, Neurogenesis, Epigenetics, Proneural

Introduction

Mash1 (Ascl1) is a key transcription factor essential during embryogenesis for the production of neural precursor cells and for promoting the commitment of these multipotent progenitors to different neuronal fates including noradrenergic, serotonergic and olfactory neurons, whilst inhibiting their astrocyte potential (Bertrand et al., 2002; Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 2004). Identified through its homology and shared function to the Achaete-Scute family in *Drosophila*, Mash1 is a member of a small group of basic helix-loop-helix proteins known as 'proneural' factors which bind to and activate target promoters through interaction with E-proteins and the E-box hexanucleotide motif (Johnson et al., 1990). Mash1 expression during development is tightly regulated (Bertrand et al., 2002; Kageyama et al., 2005) and factors responsible for the transcriptional upregulation (retinoic acid and neurotrophins) and downregulation (Hes1) have been identified (Ishibashi et al., 1995; Ito et al., 2003; Itoh et al., 1997; Shoba et al., 2002) as well as cis-acting regulatory elements within the locus (Meredith and Johnson, 2000).

Gene transcription is regulated by an integrated hierarchy of sequence elements, transcription factors and local modifications to chromatin structure (van Driel et al., 2003). In addition, the spatial organisation of chromatin within the nucleus can also influence gene expression. For example, positioning of individual gene loci relative to specific nuclear landmarks – such as heterochromatin, the nuclear periphery, chromosome territories and nuclear bodies – has been associated with different transcriptional states in a variety of cell types (Baxter et al., 2002; Gasser, 2001; Kosak and Groudine, 2004; Williams, 2003).

Replication timing during S phase is known to broadly correlate with transcriptional activity and chromatin structure: accessible chromatin including expressed genes replicates early whereas constitutive heterochromatin and some facultative heterochromatin replicates later (Azuara et al., 2003; Gilbert, 2002; Schubeler et al., 2002). We have recently shown that *Mash1* replicates late during S phase in undifferentiated ES cells, but much earlier when these cells were induced to generate neural progenitors after treatment with retinoic acid (Perry et al., 2004). These results indicate that global changes in chromatin structure of the *Mash1* locus

occur in response to neural induction. To define the full nature of such changes and how they are important for regulating *Mash1*, we have systematically compared the epigenetic status of *Mash1* in undifferentiated ES cells and ES-derived neural progenitors. Here we show that in ES cells the late-replicating, transcriptionally inactive *Mash1* locus is positioned at the nuclear periphery but that upon neural induction the locus relocates towards the nuclear interior. Repositioning of *Mash1* is neural specific and is coupled with transcriptional upregulation as well as local changes in the abundance of modified histones at the *Mash1* promoter. Given the extent of repositioning, neighbouring genes were also found to relocate towards the interior. However, evidence is provided that chromatin reorganisation initiates at the *Mash1* gene.

Results

Mash1 is transcriptionally upregulated upon neural differentiation

Efficient neural induction of OS25 ES cells was achieved using a standard protocol (Billon et al., 2002) and verified by immunofluorescence. Undifferentiated (day 0) ES cell cultures contained, on average, 90% Oct4-positive cells, whereas neural-induced cultures (day 8) contained 85% nestin-positive cells with less than 5% of cells Oct4 positive. Using this system, we have previously shown that upon neural induction *Mash1* switched from late to early replication (Perry et al., 2004). We assessed to what extent *Mash1* might be upregulated upon neural induction of ES cells. *Mash1* transcripts were not detected in undifferentiated ES cells, even after 30 cycles of RT-PCR, but were abundant in day 8 neural cells (Fig. 1A). Control ES- and neural-specific markers showed the expected pattern of expression. To determine whether very low levels of *Mash1* transcription or alternative RNA products could account for a previous report that *Mash1* can be transcribed in undifferentiated ES cells (Ying et al., 2003) we also performed semi-quantitative RT-PCR and northern blot analysis. The latter revealed a single band for *Mash1* in retinoic-acid-differentiated OS25 cells which was undetectable in undifferentiated ES cells (data not shown), whereas the RT-PCR revealed that a low level of *Mash1* can be detected in ES cells after 35 cycles: at least 125-fold lower in abundance than

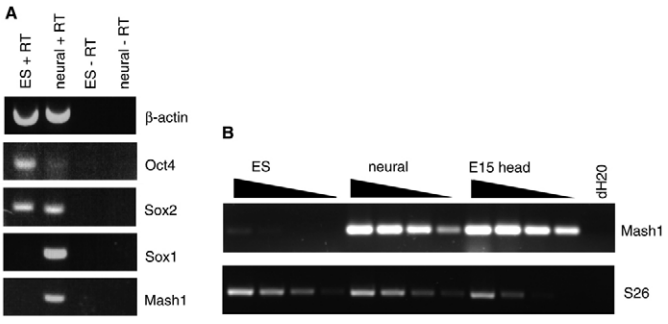

Fig. 1. Transcription analysis of *Mash1* in ES and neural cells. (A) RT-PCR of *Mash1* and control genes from OS25 ES and neural cell RNA. After 30 cycles of PCR *Mash1* is detected in the neural but not the ES sample. Markers for ES to neural differentiation, *Oct4, Sox2* and *Sox1*, show expected expression patterns. β-actin was used as a template input control. –RT, reverse-transcriptase-free negative control. (B) Semi-quantitative RT-PCR shows that transcription of *Mash1* occurs at a negligible level in ES cells but is dramatically upregulated (at least 125-fold) in neural cells. Dilutions were: 1, 1:5, 1:25, 1:125, left to right. S26, small ribosomal protein 26 template-input control. E15 head, positive control for *Mash1* expression. dH_2O, negative control.

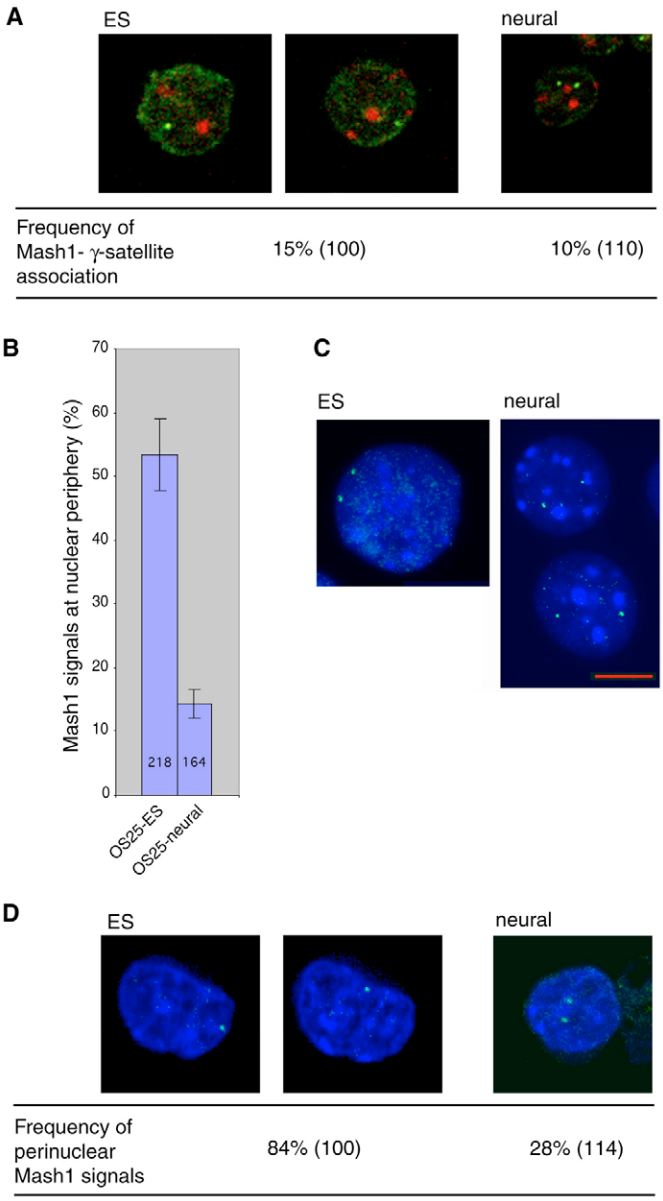

Fig. 2. Spatial relationship of the *Mash1* locus to centromeric heterochromatin and the nuclear periphery. (A) By 3D FISH we observed that the *Mash1* locus (green) is rarely associated with centromeric heterochromatin (γ-satellite probe is red) in either ES or neural cells. Numbers in brackets are FISH signal scores. (B) Frequency (mean ± s.d.) of FISH signals scored as peripheral (⩾80% of nuclear radius) in undifferentiated OS25 ES cells and differentiated OS25 neural cells. Numbers in bars are total fish signals scored. (C) Representative 2D FISH result showing *Mash1* locus (green) in OS25 ES and neural cells. Nuclei were counterstained with DAPI (blue). (D) Representative 3D FISH result showing *Mash1* locus (green) in individual *z* slices of DAPI-stained ES and neural nuclei. Frequency of perinuclear *Mash1* signals scored in 3D analysis is indicated. Numbers in brackets represent the total FISH signals scored. Bar, 5 μm.

that detected in day 8 samples (Fig. 1B). These data confirm that *Mash1* is dramatically upregulated during neural differentiation of ES cells in vitro.

The *Mash1* locus relocates in the nucleus following neural differentiation

A number of genes are recruited to centromeric heterochromatin when silent (Brown et al., 1999; Brown et al., 1997; Dernburg et al., 1996; Francastel et al., 1999; Grogan et al., 2001), whereas positioning of silent genes towards the nuclear periphery has been shown in yeast (Andrulis et al., 1998; Gasser, 2001) and mammalian nuclei (Kosak and Groudine, 2004; Kosak et al., 2002; Li et al., 2001). We investigated the spatial relationship of *Mash1* relative to both centromeric heterochromatin domains and the nuclear periphery. By 3D FISH using a probe for γ-satellite (pericentric DNA) and the *Mash1* locus (BAC RP24-130P7), we found that *Mash1* is rarely associated with pericentric heterochromatin in either ES or neural cells (Fig. 2A). Thus, late replication and transcriptional repression of *Mash1* in ES cells is not due to association with pericentric heterochromatin. We next investigated the spatial relationship of *Mash1* to the nuclear periphery using 2D analysis. The positions of *Mash1* FISH signals were defined as ratios of the nuclear radius. Values greater or equal to 0.8 were scored as peripheral (where the nuclear centre=0 and the periphery=1) (Kosak et al., 2002). We found that in ES cells *Mash1* was preferentially associated with the periphery. However, after neural differentiation the locus was significantly more often located away from the periphery (P<0.001 by the chi-square test) (Fig. 2B,C). This suggests that neural induction results in a coordinated switch in replication timing, transcriptional upregulation and relocation of the *Mash1* locus from the nuclear periphery to the nuclear interior. The repositioning of *Mash1* was also confirmed by additional 3D experiments. In 3D-preserved nuclei we observed 84% of *Mash1* signals adjacent to the nuclear periphery in ES cells whereas only 28% of signals were peripheral in ES-derived neural cells (Fig. 2D).

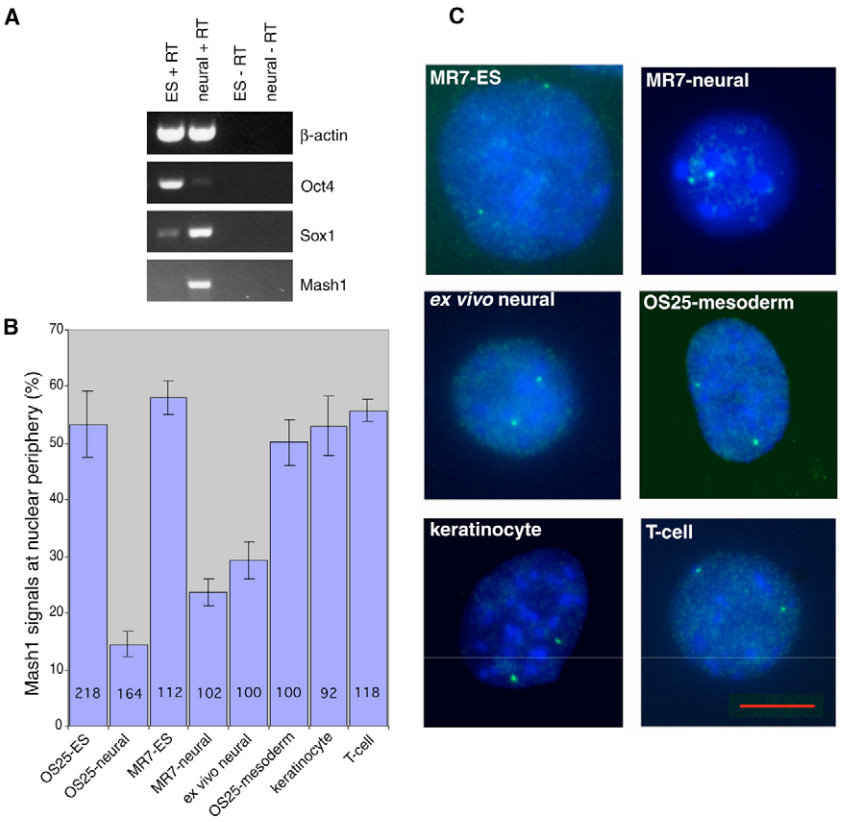

Fig. 3. Dissociation of *Mash1* from nuclear periphery is specific to neural fate. (A) RT-PCR shows *Mash1* is upregulated upon neural differentiation of MR-7 ES cells by PA6 stromal cell layer method. *Oct4* and *Sox1* controls are also shown. (B) Frequency (mean ± s.d.) of FISH signals scored as peripheral in MR-7 ES cells, PA6-differentiated MR-7 neural cells, ex vivo *Sox1*-positive neural cells, mesoderm-differentiated OS25 cells, ex vivo wild-type keratinocyte precursors and ex vivo wild-type T cells. Also shown are undifferentiated OS25 ES and neural cells from Fig. 2, for reference. Numbers in bars represent total FISH signals scored. (C) Representative 2D FISH results for each of these cell types. *Mash1* locus is green. Nuclei are counterstained with DAPI (blue). Bar, 5 μm.

Movement of *Mash1* away from the nuclear periphery is specific to the neural fate

The *Mash1* gene is known to be transcriptionally upregulated by retinoic acid (Shoba et al., 2002). Since our chosen method of neural induction involves treatment with retinoic acid we were interested in whether the movement of *Mash1* away from the nuclear periphery could be recapitulated using alternative neural differentiation methods. We differentiated MR-7 ES cells into neural cells by growth on a PA6 stromal cell layer (Kawasaki et al., 2000). By day 8 of differentiation these cultures contained 80% nestin-positive cells, and RT-PCR analysis confirmed an upregulation of *Mash1* (Fig. 3A). FISH analysis revealed that *Mash1* was located at the nuclear periphery in MR-7 ES cells and following differentiation was again relocated towards the nuclear interior. The shift was slightly less dramatic than in OS25 cells but still significant (P<0.001) (Fig. 3B,C). Movement of the *Mash1* locus away from the periphery and its transcriptional upregulation is therefore a consistent feature of neural induction of ES cells, rather than simply being a result of retinoic acid treatment. Furthermore, we extended this analysis to normal ex vivo neural progenitor cells. Neural cells, isolated from the forebrain of day 11.5-12.5 *Sox1-lacZ* mouse embryos, were labelled for *lacZ* expression and positive cells were isolated by FACS. We found that *Mash1* was located towards the nuclear interior in ex vivo neural cells (Fig. 3B,C) and furthermore was early replicating (data not shown). Again positioning of *Mash1* in ex vivo neural cells was significantly different to that in OS25 ES cells (P<0.001).

To address whether the spatial relocation of *Mash1* was specific for neural commitment or results from generalised changes associated with ES cell differentiation we examined *Mash1* in a variety of non-neural cells. OS25 ES cells were differentiated by growth on collagen IV-coated plates without

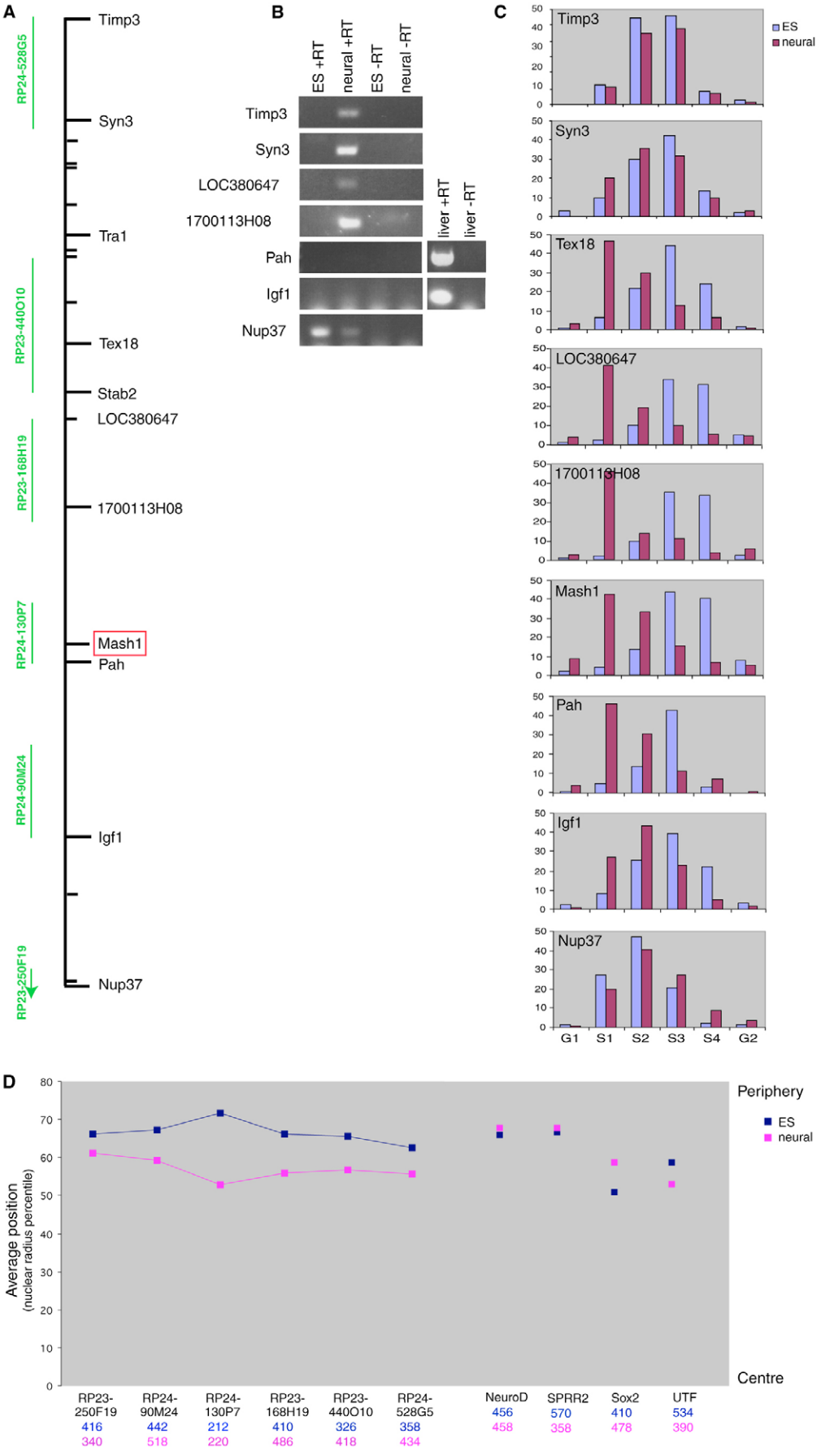

Fig. 4. The genomic environment of *Mash1*. (A) A 2 Mb region surrounding the *Mash1* gene on chromosome 10, with neighbouring genes annotated. (B) RT-PCR analysis of genes surrounding *Mash1* in OS25 ES and NE cells. (C) Replication timing of genes surrounding *Mash1* in ES and NE cells. Bar charts show the abundance of nascent DNA in each S-phase fraction (as a percentage of the total). (D) Positions of the 2 Mb region probes relative to nuclear periphery (probe positions shown annotated in A). Also shown are the positions of silent, late replicating genes, *Neurod* (NeuroD) and *Sprr2* (SPRR2) and active, early replicating genes, *Sox2* (Sox2) and *Utf1* (UTF) as controls. Numbers beneath probes represent FISH signals scored in high-throughput analysis (blue, ES cells; pink, neural cells).

LIF. Mesodermal cells ($FLK^+/Ecad^-$) were then purified from the differentiated population by FACS as described previously (Fraser et al., 2003). In these cells *Mash1* was found to remain at the nuclear periphery. *Mash1* was also located at the nuclear periphery in ex vivo keratinocyte progenitors and ex vivo T cells (Fig. 3B,C). *P* values were not significant for these three cell types compared to OS25 ES cells (*P*=1, 0.599 and 0.299 for OS25 ES vs mesoderm, keratinocytes and T cells, respectively) confirming that repositioning of *Mash1* to the nuclear interior is selective for neural-committed cells.

Epigenetic changes are focussed at the *Mash1* locus

As relocation of *Mash1* appears to be specific for neural cells, we asked whether neural-specific epigenetic changes are nucleated at the *Mash1* locus or are a passive reflection of genomic events at a neighbouring gene. For example, the entire chromosome territory may move position during neural differentiation, or the regulation of upstream or downstream genes may influence positioning. In silico analysis of a 2 Mb region surrounding the *Mash1* locus on chromosome 10 did not reveal any neural-specific genes close to *Mash1* (Fig. 4A). RT-PCR analysis of the genes around *Mash1* show an interesting pattern of expression during ES to neural differentiation (Fig. 4B). Strikingly, the *Pah* gene which is just 30 kb telomeric to *Mash1* is silent in both ES and neural cells and so too is the *Igf1* gene, a further 300 kb telomeric. *Nup37* on the other hand is expressed robustly in ES cells and is also expressed in neural progenitors. Centromeric to *Mash1* however all four genes analysed were transcriptionally upregulated in response to neural induction, in common with *Mash1*. Given that none of these genes are known to be neural specific, it seems unlikely that they are the targets for dominant chromatin cues involved in repositioning *Mash1*, although of course we cannot completely rule this out.

To examine how far around *Mash1* the replication timing switch and nuclear repositioning extends, we performed a 'genome walk' either side of *Mash1*, from *Timp3* to *Nup37*. As shown in Fig. 4C, the difference in replication timing between ES and neural progenitors was most marked in the *Mash1-LOC380647* (*Gm1554*) region but extended to 1.2 Mb centromeric (*Timp3*) and 0.65 Mb telomeric (*Nup37*) where replication was at mid S phase in both cell types. To look at nuclear repositioning of genes around *Mash1*, given the large number of loci to study and the potential need to be able to detect subtle movements, we used a high-throughput, semi-automated system previously reported (Roix et al., 2003). The map positions of the probes used for this analysis are shown in Fig. 4A (indicated in green). We looked at loci at distances from *Mash1* up to the point of no replication timing change in both directions. All six probes covering the 2 Mb region around the *Mash1* locus showed significant movement away from the nuclear periphery towards a more internal location (Fig. 4D). These data indicate that despite movement being specific to neural cells (and therefore probably coordinated at *Mash1*), the region as a whole relocates during neural commitment and confirms that transcriptional status of certain genes is independent of position relative to the nuclear periphery (*Pah, Igf1, Nup37*). Interestingly, a similar analysis of two unlinked genes: *Neurod* (involved in neurogenesis, located on chromosome 2C3) and *Sprr2a* (involved in epidermal differentiation, located on chromosome 3F1), which like *Mash1* are inactive and late replicating in undifferentiated ES cells, showed that these loci also occupied a peripheral position. In contrast, the expressed and early replicating genes *Sox2* and *Utf1* were more centrally located in undifferentiated ES cell nuclei (Fig. 4D). These data raise the possibility that positioning of developmentally regulated genes towards the nuclear periphery might be important for maintaining their silent state and thus the pluripotency of ES cells. In day 8 differentiated neural cells both *Neurod* and *Sprr2a* are inactive and late replicating and, consistent with this, both remain closely associated with the nuclear periphery. *Sox2* and *Utf1* on the other hand, which are both early replicating in neural cells, do not associate with the periphery (Fig. 4D).

To define the chromatin changes at the *Mash1* promoter that accompany neural induction, we performed chromatin immunoprecipitation (ChIP) analysis. The specificity of these changes was assessed by comparing *Mash1* to its two nearest neighbouring genes, *Pah* and *1700113H08rik*. In undifferentiated OS25 ES cells we observed abundant Me_3H3K27 at the *Mash1* promoter. Upon neural differentiation the level of Me_3H3K27 declined and we observed a reciprocal increase in levels of H3K9 acetylation (Fig. 5A). Although both of these modifications, AcH3K9 and Me_3H3K27 showed some change at the neighbouring *Pah* and *1700113H08rik* genes, neither showed levels comparable to *Mash1* giving evidence that these chromatin modifications associated with neural induction are orchestrated at, and specific to, *Mash1*.

Low levels of both Me_3H3K9 and Me_3H4K20 at all three genes, compared with pericentromeric heterochromatin (positive control; data not shown) indicates that neither are likely to be involved in the silencing and peripheral localisation of *Mash1*.

Peripheral positioning of *Mash1* does not require HMTases or DNMTases

The abundance of H3K27 methylation at the *Mash1* promoter in ES cells, suggests that this modification could be important for facultative heterochromatin formation and tethering *Mash1* to the nuclear periphery. To test this we examined the positioning of *Mash1* in ES cells lacking the Eed/Ezh2 HMTase complex, which is required for H3K27 methylation (Montgomery et al., 2005; Silva et al., 2003). In these cells, *Mash1* was preferentially positioned at the nuclear periphery, similar to the OS25 and MR7 ES cells (Fig. 5B,C). To investigate whether other chromatin-silencing factors might be responsible for tethering the *Mash1* locus to the nuclear periphery we analysed a number of other ES cell lines targeted for the following candidates: the HMTases, Suv39h-1 and G9a and the DNA methyltransferases, Dnmt1 and Dnmt3a and 3b. None of the ES cell lines analysed showed *Mash1* signals in a preferentially non-peripheral location in the nucleus (Fig. 5B,C), i.e. when compared with OS25 ES cells, none were significantly different (*P*=0.426, 0.637, 0.326, 0.619 and 0.619 for ES vs $Eed^{-/-}$, $G9a^{-/-}$, $SuV39h1^{-/-}$, $Dnmt1^{-/-}$ and $Dnmt3ab^{-/-}$, respectively). The results therefore suggest that these chromatin silencing factors are not uniquely required for tethering *Mash1* to the nuclear periphery.

Discussion

Mash1 relocates in response to neural induction

Dissecting the epigenetic mechanisms regulating key developmental genes is important for understanding both how

ES cell pluripotency is maintained, and how cell fate is assigned in the developing embryo. Using ES cells as a model system, we characterised the epigenetic changes that occur at the *Mash1* locus in response to neural commitment. From a previous survey of approximately 50 transcription factor genes, *Mash1* was shown to be one of only three that replicates late in ES cells (Perry et al., 2004). Upon neural induction of ES cells *Mash1* was shown to switch from late replication to early and we show here that in parallel *Mash1* is transcriptionally upregulated, acquires H3K9 acetylation across its promoter, declines in H3K27 methylation and is relocated from the nuclear periphery towards the interior.

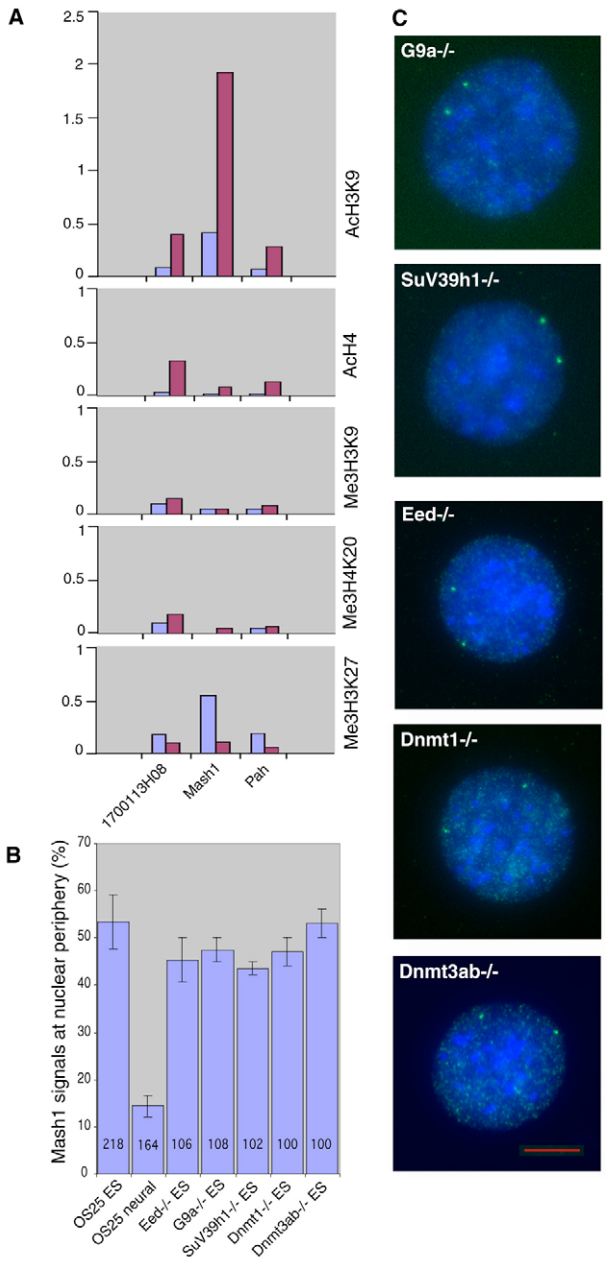

Fig. 5. Chromatin organisation at *Mash1*. (A) Histone modifications of *Mash1* and neighbouring genes in ES and neural cells. Bars show relative levels of real-time PCR products amplified from ES (pink) and NE (blue) genomic DNA following ChIP with antibodies to AcH3K9, pan-AcH4, Me_3H3K9, Me_3H3K20 and Me_3H3K27. Results are normalized to histone H3 ChIP PCR levels. (B) *Mash1* positioning in ES cells lacking chromatin-silencing factors. Bar chart shows number of *Mash1* FISH signals scored as peripheral in $Eed^{-/-}$, $G9a^{-/-}$, $SuV39h1^{-/-}$, $Dnmt1^{-/-}$ and $Dnmt3ab^{-/-}$ ES cells. Also shown are OS25 ES and neural cells from Fig. 2, for reference. Numbers in bars represent total FISH signals scored. (C) Representative 2D FISH results for each of these knockout ES cells. *Mash1* locus, green. Nuclei counterstained with DAPI (blue). Bar, 5 μm.

We demonstrate that *Mash1* repositioning is selective for neural differentiation because *Mash1* was centrally located in ES-derived neural cells and primary neural cells but not in T cells, keratinocytes or ES-derived mesodermal cells. This neural specificity argues that general features of differentiation such as the reorganisation of large blocks of heterochromatin are unlikely in themselves to be the cause of *Mash1* relocation. The finding that changes in histone modifications were prominent at the *Mash1* promoter compared with its neighbouring genes and that maximal changes in replication timing were centred at the *Mash1-LOC380647* region, argue that these epigenetic changes are nucleated at *Mash1*. A recent report that loci which change replication timing during ES differentiation are often AT rich and LINE dense (Hiratani et al., 2004) fits well with the occurrence of an approximately 300 kb cluster of full-length LINE elements, spanning the region from *Mash1* to *LOC380647*.

What might constrain *Mash1* at the periphery in ES cells? In the *Drosophila* embryo the genome is non-randomly associated with the nuclear periphery (tethered) at regular intervals (Marshall, 2002; Marshall et al., 1996). Consistent with this, live-cell analysis of chromatin dynamics in human nuclei has revealed that loci nearer to the nuclear periphery have a smaller radius of confinement (indicating restraint by tethering) than loci positioned towards the interior (Chubb et al., 2002). Our data show that *Mash1* is associated with the nuclear periphery in undifferentiated ES cells and that, in these cells, H3K27 methylation is readily detected at the *Mash1* promoter. Although this result suggests that facultative heterochromatin could play a role in *Mash1* tethering, analysis of a panel of ES cells deficient for specific chromatin silencing factors – including Eed, a component of the H3K27-specific HMTase – did not reveal any major changes in *Mash1* location. This could either mean that none of these chromatin modifications are required, or that the cells are able to compensate for loss of an individual factor by alternative mechanisms (redundancy). Alternatively, the nuclear periphery may represent a 'default' location for *Mash1* in ES cells, where relocation is a response to locus activation. In this regard, it has previously been shown that the transcriptional activator VP16 can drive the spatial relocation of a *lac*-operator repeat array from a preferentially peripheral nuclear location towards the interior (Tumbar et al., 1999). Also, in lymphocytes and neuroblastoma cells the cystic fibrosis gene (*CFTR*) relocates away from a poorly acetylated chromatin environment of the nuclear periphery, to an internal position in response to treatment with the histone deacetylase (HDAC) inhibitor trichostatin A (Zink et al., 2004). Although the observation that neural differentiation results in increased H3K9 acetylation across the *Mash1* promoter, could fit this interpretation, HDAC inhibitors dramatically affect the global organisation of constitutive heterochromatin in differentiating cells as well as

other cell types (Taddei et al., 2001) which complicates their use in our present model system.

Peripheral positioning and transcriptional activity

Recently, *CFTR* and two neighbouring genes that are differentially expressed were shown to localise to discrete areas relative to the nuclear periphery, in a transcription-dependent manner (Zink et al., 2004). In our study, repositioning of *Mash1* resulted in the relocation and replication time switching of a number of neighbouring genes. Two of these, *Pah* and *Igf1*, remained transcriptionally silent in both ES and ES-derived neural cells. This shows that certain epigenetic features of accessible chromatin such as a favourable nuclear position or time of DNA replication, are insufficient in themselves to drive gene expression: a conclusion that has also been highlighted by studies of the α-globin locus in non-erythroid cells (Brown et al., 2001; Smith and Higgs, 1999). Further from *Mash1* – 1.2 Mb centromeric (*Timp3*) and 0.65 Mb telomeric (*Nup37*) – replication was in middle S phase in both cell types. *Nup37* was transcribed in both cell types, however, *Timp3* was silent in ES cells and upregulated in neural cells, indicating that change in replication timing is not a general feature of changing transcriptional status.

The fact that *Pah* and *Igf1* remain silent, whereas other *Mash1* neighbouring genes such as *Timp3, Syn3, LOC380647* and *1700113H08rik* are coordinately upregulated in response to neural induction, could indicate the presence of a so-called boundary element or insulator (Labrador and Corces, 2002) between *Mash1* and *Pah*. Although no potential CTCF binding sites have yet been identified, a BLAST analysis of the 30 kb separating the these genes revealed a region of approximately 1.2 kb which was highly conserved between human and mouse (87% conserved sequence, located 6 kb upstream of the *Mash1* promoter) that contains a functional enhancer element (Meredith and Johnson, 2000; Verma-Kurvari et al., 1998).

The nuclear periphery is generally considered a region of transcriptional repression, being enriched for late-replicating, gene-poor, hypoacetylated chromatin (O'Keefe et al., 1992; Croft et al., 1999; Ferreira et al., 1997; Sadoni et al., 1999; Tanabe et al., 2002). Positioning of silent genes at the nuclear periphery has been shown in yeast (Andrulis et al., 1998; Gasser, 2001) and mammalian nuclei (Kosak et al., 2002; Li et al., 2001), whereas actively transcribed genes and early replicating DNA tend to locate towards the nuclear interior (O'Keefe et al., 1992; Carter et al., 1993; Kosak and Groudine, 2004). In yeast, telomeres cluster at the nuclear periphery and recruit SIR proteins, known to be involved in silencing (Cockell et al., 1995; Cockell and Gasser, 1999). By artificially tethering a reporter gene to the yeast nuclear periphery, silencing of the reporter was induced and shown to be SIR dependent (Andrulis et al., 1998), providing a clear mechanism in which loci are both repressed and physically tethered to the nuclear periphery. Recent studies indicate a more complex role of the nuclear periphery in gene regulation. A genome-wide analysis in yeast has shown that nuclear pore proteins associate with a subset of highly transcribed genes (Casolari et al., 2004). The nuclear pore complex has also been implicated in boundary activity (Labrador and Corces, 2002), a finding supported by the observation that the *gypsy* insulator element localises to the periphery in *Drosophila* nuclei (Gerasimova et al., 2000). In mammalian cells, however, a role for the nuclear periphery in actively regulating gene repression remains unproven. We have recently shown that the interferon-γ gene was constitutively located at the nuclear periphery in T cells, apparently irrespective of its expression (Hewitt et al., 2004). Here we show that certain genes (*Pah, Igf1)* change their location in differentiating ES cells despite remaining transcriptionally inactive. Probably these closely linked genes are simple 'passengers' of chromatin changes that are centred at the *Mash1* locus and that drive spatial repositioning of the entire region. Whatever the explanation, the demonstration that two other developmentally important but unlinked genes – *Neurod* and *Sprr2a* (selected as representatives of inactive and late-replicating genes in undifferentiated ES cells) – in addition to *Mash1*, both localise at the nuclear periphery in undifferentiated ES cells is intriguing. These data suggest that the nuclear periphery could function as a repressive compartment within undifferentiated ES cells, important for maintaining their uncommitted status.

Materials and Methods

Cell culture

OS25 ES cells, in which the β-geo gene is inserted at the *Sox2* locus and the hygromycin-thymidine kinase gene is inserted at the *Oct4* locus, were cultured as previously described (Billon et al., 2002; Perry et al., 2004). MR-7 ES cells were maintained on irradiated primary embryonic fibroblasts plated on gelatin-coated dishes in G-MEM supplemented with 1% FCS (Globepharm), 10% KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol (2-ME), penicillin-streptomycin (GIBCO-BRL) and 1000 U/ml LIF (Chemicon). Sox1-positive ex vivo neural cells (a kind gift from C. Andonaidou and R. Lovell-Badge, NIMR, London, UK) were isolated from day 11.5-12.5 *Sox1-lacZ* mouse embryos by dissociating forebrains into single cell suspension and labelling *lacZ*-positive cells using the DetectaGene Green CMFDG *lacZ* Gene Expression kit (Molecular Probes), followed by FACS. T lymphocytes were isolated from lymph nodes of 6- to 8-week-old C57BL/6 mice and were cultured for 3-4 days in IMDM supplemented with 10% FCS, 2.5×10-5 M 2-mercaptoethanol and antibiotics on culture plates coated with 2 μg/ml anti-TCRb (H57-597; BD Pharmingen) and 10 μg/ml ati-CD28 (BD Pharmingen) with Il2 (1 ng/ml). Primary keratinocytes were isolated from 3-day-old mice as described previously (Hennings, 1994) and cultured in complete 'low-calcium FAD' (0.1 mM Ca^{2+}) (one part Ham's F12 and three parts Dulbecco's modified Eagle's medium) supplemented with FBS and growth factors (Hobbs et al., 2004). Cells were plated on collagen coated plates and were grown as attached, undifferentiated precursor keratinocytes.

ES cell differentiation

OS25 ES cells were differentiated into neural ectoderm as previously described (Billon et al., 2002; Perry et al., 2004). Briefly, cells were trypsinised and plated onto 10 cm bacterial dishes without LIF to allow embryoid bodies to form. 1 μM retinoic acid was added on day 4, removed on day 6 and replaced with DMEM-F12 (containing N2) and neurobasal media (containing B27) in a 1:1 ratio. From day 6-8 selection for expression of *Sox2* and against expression of *Oct4* was achieved using G-418 (100 μg/ml) and gancyclovir (2.5 μM) respectively. On day 8, cells were harvested for RNA extraction, immunofluorescence as previously described (Perry et al., 2004) and for FISH fixation.

MR-7 ES cells were differentiated into neural cells by plating on irradiated PA6 stromal cells in G-MEM medium supplemented with 10% KSR, 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-ME (Kawasaki et al., 2000). Again, day 8 cells were harvested for FISH fixation, immunofluorescence and RNA extraction.

Differentiation of OS25 cells into mesoderm was carried out as previously described (Fraser et al., 2003) with minor modifications. Cells were trypsinised and plated at low density (0.5×10^3 cells/cm^2) on collagen IV-coated plates in differentiation medium without LIF. After 5 days cells were harvested using cell dissociation buffer (Sigma) and labelled with PE-conjugated anti-FLK and FITC-conjugated anti-E-cadherin (Pharmingen). FLK^+/E-$cadherin^-$ mesoderm cells were collected by FACS for FISH fixation.

RT-PCR

RNA extraction was performed using RNAzol B (Biogenesis). Cells were resuspended in 0.2 ml RNAzol per 10^6 cells, mixed thoroughly, and then subjected to chloroform extraction and isopropanol precipitation before resuspension in RNAse-free water. One μg total RNA was then reverse transcribed using Superscript™ First Strand Synthesis kit (Invitrogen) and cDNAs of interest were

PCR amplified in 20 μl reaction containing 2 U Taq polymerase (New England Biolabs) and 0.5 μM of each primer. The following cycling conditions were used: 94°C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, finishing with 72°C for 5 minutes. For semi-quantitative RT-PCR, cDNA from OS25 cells (undifferentiated and day 8 differentiated), and mouse E15 embryonic heads was serially diluted and 2 μl of 1:1, 1:5, 1:25 and 1:125 dilutions were amplified by 35 cycles of PCR. Primer sequences are available on request.

Fluorescence in situ hybridisation (FISH)

BAC probes for FISH were checked for the presence of the appropriate gene by PCR and then labelled with digoxygenin using the Nick Translation kit (Invitrogen). Specific hybridisation of BACs was confirmed by metaphase FISH. For 2D Interphase, FISH cells were harvested by trypsinisation, and hypotonically treated in 75 mM KCl for 5 minutes at room temperature before fixing in ice-cold methanol: acetic acid (3:1). Fixed cells were dropped onto slides, denatured, hybridised and washed as previously described (Williams et al., 2002). For 3D FISH cells were harvested, washed in PBS and attached to poly-L-lysine-coated coverslips. Fixation, denaturation, hybridisation and washing were all carried out as previously described (Brown et al., 1997).

Microscopy and measurements

FISH on 3D-preserved nuclei was viewed under a Leica laser-scanning confocal microscope, using a 100× oil-immersion objective. Optical slices across the *z*-axis of nuclei were captured every 0.25 μm to create *z*-stacks for analysis. *Mash1* was scored as 'associated' with γ-satellite when the two signals were touching or overlapping (see Williams et al., 2002). FISH on 2D-fixed nuclei was viewed under a Leica epifluorescence microscope, using a 100× oil-immersion objective. Images were captured with a CCD camera and analysed using IP lab software. The position of loci relative to the nuclear periphery was determined by measuring the distance from the nuclear centroid to the FISH signal as a ratio of the nuclear radius (radius=distance of nuclear centroid to nuclear periphery through the FISH signal). FISH signals at ⩾80% of nuclear radius were considered peripheral (Kosak et al., 2002). For each cell type at least two experiments were performed and approximately 100 FISH signals were scored. The chi-square test for significance was performed on the pooled totals for each cell type. High-throughput analysis of nuclear positioning was performed as previously described (Roix et al., 2003), and for these the Wilcoxon–Mann-Whitney test for significance was performed.

Replication timing analysis

OS25 cells were BrdU labelled, fixed in 70% ethanol and sorted into cell-cycle fractions as previously described (Azuara et al., 2003; Perry et al., 2004). BrdU-labelled nascent DNA was isolated by immunoprecipitation and the abundance of nascent DNA in each fraction for the genes of interest was determined by real-time PCR (primer sequences available on request).

Chromatin immunoprecipitation (ChIP) analysis

OS25 cells were harvested on day 0 (ES) and day 8 (neural) of differentiation and processed for ChIP analysis as previously described (Baxter et al., 2004). Briefly, 140 μg chromatin was immunoprecipitated with 2 μl anti-histone H3 (input control, Abcam 1791), 3 μl anti-acetyl-histone H3K9 (Upstate, 07-352), 5 μl anti-acetyl-histone H4 (Serotec, AHP418), 5 μl anti-trimethyl-histone H3K9 and anti-trimethyl-histone H4K20 (both from Thomas Jenuwein) and 2 μl rabbit anti-mouse-IgG antiserum (negative control, Dako). DNA was then eluted from immune complexes and quantification of recovered DNA was performed using real-time PCR (primer sequences available on request).

We thank Francois Guillemot for *Mash1* BAC, RP24-130P7, Cynthia Andoniadou and Robin Lovell-Badge for the *Sox1/LacZ*-positive primary neural cells and Simon Broad for the mouse embryonic keratinocyte precursors. We thank Bernard Ramsahoye and En Li, Yoichi Shinkai, Thomas Jenuwein and Rosalind John for the Dnmt, G9a, SuV39h-1 and Eed mutant ES cell lines, respectively. We thank Mikhail Spivakov for help with the replication timing analysis. This work was supported by the Medical Research Council, UK.

References

Andrulis, E. D., Neiman, A. M., Zappulla, D. C. and Sternglanz, R. (1998). Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394**, 592-595.

Azuara, V., Brown, K. E., Williams, R. R., Webb, N., Dillon, N., Festenstein, R., Buckle, V., Merkenschlager, M. and Fisher, A. G. (2003). Heritable gene silencing in lymphocytes delays chromatid resolution without affecting the timing of DNA replication. *Nat. Cell Biol.* **5**, 668-674.

Baxter, J., Merkenschlager, M. and Fisher, A. G. (2002). Nuclear organisation and gene expression. *Curr. Opin. Cell Biol.* **14**, 372-376.

Baxter, J., Sauer, S., Peters, A., John, R., Williams, R., Caparros, M. L., Arney, K., Otte, A., Jenuwein, T., Merkenschlager, M. et al. (2004). Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. *EMBO J.* **23**, 4462-4472.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Billon, N., Jolicoeur, C., Ying, Q. L., Smith, A. and Raff, M. (2002). Normal timing of oligodendrocyte development from genetically engineered, lineage-selectable mouse ES cells. *J. Cell Sci.* **115**, 3657-3665.

Brown, K. E., Guest, S. S., Smale, S. T., Hahm, K., Merkenschlager, M. and Fisher, A. G. (1997). Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* **91**, 845-854.

Brown, K. E., Baxter, J., Graf, D., Merkenschlager, M. and Fisher, A. G. (1999). Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Mol. Cell* **3**, 207-217.

Brown, K. E., Amoils, S., Horn, J. M., Buckle, V. J., Higgs, D. R., Merkenschlager, M. and Fisher, A. G. (2001). Expression of alpha- and beta-globin genes occurs within different nuclear domains in haemopoietic cells. *Nat. Cell Biol.* **3**, 602-626.

Carter, K. C., Bowman, D., Carrington, W., Fogarty, K., McNeil, J. A., Fay, F. S. and Lawrence, J. B. (1993). A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. *Science* **259**, 1330-1335.

Casolari, J. M., Brown, C. R., Komili, S., West, J., Hieronymus, H. and Silver, P. A. (2004). Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. *Cell* **117**, 427-439.

Chubb, J. R., Boyle, S., Perry, P. and Bickmore, W. A. (2002). Chromatin motion is constrained by association with nuclear compartments in human cells. *Curr. Biol.* **12**, 439-445.

Cockell, M. and Gasser, S. M. (1999). Nuclear compartments and gene regulation. *Curr. Opin. Genet. Dev.* **9**, 199-205.

Cockell, M., Palladino, F., Laroche, T., Kyrion, G., Liu, C., Lustig, A. J. and Gasser, S. M. (1995). Thecarboxy termini of Sir4 and Rap1 affect Sir3 localization: evidence for a multicomponentcomplex required for yeast telomeric silencing. *J. Cell Biol.* **129**, 909-924.

Croft, J. A., Bridger, J. M., Boyle, S., Perry, P., Teague, P. and Bickmore, W. A. (1999). Differences in the localization and morphology of chromosomes in the human nucleus. *J. Cell Biol.* **145**, 1119-1131.

Dernburg, A. F., Broman, K. W., Fung, J. C., Marshall, W. F., Philips, J., Agard, D. A. and Sedat, J. W. (1996). Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell* **85**, 745-759.

Ferreira, J., Paolella, G., Ramos, C. and Lamond, A. I. (1997). Spatial organization of large-scale chromatin domains in the nucleus: a magnified view of single chromosome territories. *J. Cell Biol.* **139**, 1597-1610.

Francastel, C., Walters, M. C., Groudine, M. and Martin, D. I. (1999). A functional enhancer suppresses silencing of a transgene and prevents its localization close to centrometric heterochromatin. *Cell* **99**, 259-269.

Fraser, S. T., Yamashita, J., Jakt, L. M., Okada, M., Ogawa, M. and Nishikawa, S. (2003). In vitro differentiation of mouse embryonic stem cells: hematopoietic and vascular cell types. *Methods Enzymol.* **365**, 59-72.

Gasser, S. M. (2001). Positions of potential: nuclear organization and gene expression. *Cell* **104**, 639-642.

Gerasimova, T. I., Byrd, K. and Corces, V. G. (2000). A chromatin insulator determines the nuclear localization of DNA. *Mol. Cell* **6**, 1025-1035.

Gilbert, D. M. (2002). Replication timing and transcriptional control: beyond cause and effect. *Curr. Opin. Cell Biol.* **14**, 377-383.

Grogan, J. L., Mohrs, M., Harmon, B., Lacy, D. A., Sedat, J. W. and Locksley, R. M. (2001). Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* **14**, 205-215.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463-476.

Hennings, H. (1994). Primary culture of keratinocytes from newborn mouse epidermis in medium with lowered levels of Ca2+. Cambridge: Cambridge University Press.

Hewitt, S. L., High, F. A., Reiner, S. L., Fisher, A. G. and Merkenschlager, M. (2004). Nuclear repositioning marks the selective exclusion of lineage-inappropriate transcription factor loci during T helper cell differentiation. *Eur. J. Immunol.* **34**, 3604-3613.

Hiratani, I., Leskovar, A. and Gilbert, D. M. (2004). Differentiation-induced replication-timing changes are restricted to AT-rich/long interspersed nuclear element (LINE)-rich isochores. *Proc. Natl. Acad. Sci. USA* **101**, 16861-16866.

Hirsch, M. R., Tiveron, M. C., Guillemot, F., Brunet, J. F. and Goridis, C. (1998). Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. *Development* **125**, 599-608.

Hobbs, R. M., Silva-Vargas, V., Groves, R. and Watt, F. M. (2004). Expression of activated MEK1 in differentiating epidermal cells is sufficient to generate hyperproliferative and inflammatory skin lesions. *J. Invest. Dermatol.* **123**, 503-515.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R. and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev.* **9**, 3136-3148.

Ito, H., Nakajima, A., Nomoto, H. and Furukawa, S. (2003). Neurotrophins facilitate neuronal differentiation of cultured neural stem cells via induction of mRNA expression of basic helix-loop-helix transcription factors Mash1 and Math1. *J. Neurosci. Res.* **71**, 648-658.

Itoh, F., Nakane, T. and Chiba, S. (1997). Gene expression of MASH-1, MATH-1,

neuroD and NSCL-2, basic helix-loop-helix proteins, during neural differentiation in P19 embryonal carcinoma cells. *Tohoku J. Exp. Med.* **182**, 327-336.

Johnson, J. E., Birren, S. J. and Anderson, D. J. (1990). Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. *Nature* **346**, 858-861.

Kageyama, R., Ohtsuka, T., Hatakeyama, J. and Ohsawa, R. (2005). Roles of bHLH genes in neural stem cell differentiation. *Exp. Cell Res.* **306**, 343-348.

Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Nishikawa, S. I. and Sasai, Y. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* **28**, 31-40.

Kosak, S. T. and Groudine, M. (2004). Form follows function: The genomic organization of cellular differentiation. *Genes Dev.* **18**, 1371-1384.

Kosak, S. T., Skok, J. A., Medina, K. L., Riblet, R., Le Beau, M. M., Fisher, A. G. and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science* **296**, 158-162.

Labrador, M. and Corces, V. G. (2002). Setting the boundaries of chromatin domains and nuclear organization. *Cell* **111**, 151-154.

Li, F., Chen, J., Izumi, M., Butler, M. C., Keezer, S. M. and Gilbert, D. M. (2001). The replication timing program of the Chinese hamster beta-globin locus is established coincident with its repositioning near peripheral heterochromatin in early G1 phase. *J. Cell Biol.* **154**, 283-292.

Marshall, W. F. (2002). Order and disorder in the nucleus. *Curr. Biol.* **12**, R185-R192.

Marshall, W. F., Dernburg, A. F., Harmon, B., Agard, D. A. and Sedat, J. W. (1996). Specific interactions of chromatin with the nuclear envelope: positional determination within the nucleus in Drosophila melanogaster. *Mol. Biol. Cell* **7**, 825-842.

Meredith, A. and Johnson, J. E. (2000). Negative autoregulation of Mash1 expression in CNS development. *Dev. Biol.* **222**, 336-346.

Montgomery, N. D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S. J., Otte, A. P. and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. *Curr. Biol.* **15**, 942-947.

O'Keefe, R. T., Henderson, S. C. and Spector, D. L. (1992). Dynamic organization of DNA replication inmammalian cell nuclei: spatially and temporally defined replication of chromosome-specific alpha-satellite DNA sequences. *J. Cell Biol.* **116**, 1095-1110.

Pattyn, A., Simplicio, N., van Doorninck, J. H., Goridis, C., Guillemot, F. and Brunet, J. F. (2004). Ascl1/Mash1 is required for the development of central serotonergic neurons. *Nat. Neurosci.* **7**, 589-595.

Perry, P., Sauer, S., Billon, N., Richardson, W. D., Spivakov, M., Warnes, G., Livesey, F. J., Merkenschlager, M., Fisher, A. G. and Azuara, V. (2004). A dynamic switch in the replication timing of key regulator genes in embryonic stem cells upon neural induction. *Cell Cycle* **3**, 1645-1650.

Roix, J. J., McQueen, P. G., Munson, P. J., Parada, L. A. and Misteli, T. (2003). Spatial proximity of translocation-prone gene loci in human lymphomas. *Nat. Genet.* **34**, 287-291.

Sadoni, N., Langer, S., Fauth, C., Bernardi, G., Cremer, T., Turner, B. M. and Zink, D. (1999). Nuclear organization of mammalian genomes. Polar chromosome territories build up functionally distinct higher order compartments. *J. Cell Biol.* **146**, 1211-1226.

Schubeler, D., Scalzo, D., Kooperberg, C., van Steensel, B., Delrow, J. and Groudine, M. (2002). Genome-wide DNA replication profile for Drosophila melanogaster: a link between transcription and replication timing. *Nat. Genet.* **32**, 438-442.

Shoba, T., Dheen, S. T. and Tay, S. S. (2002). Retinoic acid influences the expression of the neuronal regulatory genes Mash-1 and c-ret in the developing rat heart. *Neurosci. Lett.* **318**, 129-132.

Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T. B., Webster, Z., Peters, A. H., Jenuwein, T., Otte, A. P. and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. *Dev. Cell* **4**, 481-495.

Smith, Z. E. and Higgs, D. R. (1999). The pattern of replication at a human telomeric region (16p13.3): its relationship to chromosome structure and gene expression. *Hum. Mol. Genet.* **8**, 1373-1386.

Taddei, A., Maison, C., Roche, D. and Almouzni, G. (2001). Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases. *Nat. Cell Biol.* **3**, 114-120.

Tanabe, H., Muller, S., Neusser, M., von Hase, J., Calcagno, E., Cremer, M., Solovei, I., Cremer, C. and Cremer, T. (2002). Evolutionary conservation of chromosome territory arrangements in cell nuclei from higher primates. *Proc. Natl. Acad. Sci. USA* **99**, 4424-4429.

Tumbar, T., Sudlow, G. and Belmont, A. S. (1999). Large-scale chromatin unfolding and remodeling induced by VP16 acidic activation domain. *J. Cell Biol.* **145**, 1341-1354.

van Driel, R., Fransz, P. F. and Verschure, P. J. (2003). The eukaryotic genome: a system regulated at different hierarchical levels. *J. Cell Sci.* **116**, 4067-4075.

Verma-Kurvari, S., Savage, T., Smith, D. and Johnson, J. E. (1998). Multiple elements regulate Mash1 expression in the developing CNS. *Dev. Biol.* **197**, 106-116.

Williams, R. R. (2003). Transcription and the territory: the ins and outs of gene positioning. *Trends Genet.* **19**, 298-302.

Williams, R. R., Broad, S., Sheer, D. and Ragoussis, J. (2002). Subchromosomal positioning of the epidermal differentiation complex (EDC) in keratinocyte and lymphoblast interphase nuclei. *Exp. Cell Res.* **272**, 163-175.

Ying, Q. L., Nichols, J., Chambers, I. and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281-292.

Zink, D., Amaral, M. D., Englmann, A., Lang, S., Clarke, L. A., Rudolph, C., Alt, F., Luther, K., Braz, C., Sadoni, N. et al. (2004). Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei. *J. Cell Biol.* **166**, 815-825.